



Low molecular weight organic anions (carboxylates) increase microbial activity and alter microbial community composition in uncontaminated and diesel contaminated soil

5 Belinda C. Martin¹, Suman J. George², Charles A. Price¹, Esmail Shahsavari³, Andrew S. Ball³, Mark Tibbett⁴, Megan H. Ryan¹

¹Schools of Plant Biology, The University of Western Australia, Crawley WA, 6009, Australia

²Earth and Environment, The University of Western Australia, Crawley WA, 6009, Australia

10 ³Centre for Environmental Sustainability and Bioremediation, School of Applied Sciences, RMIT University, Melbourne VIC, 3082, Australia

⁴Centre for Agri-Environmental Research, School of Agriculture Policy and Development, University of Reading, Berkshire, RG6 6AR, United Kingdom

Correspondence to: Belinda C. Martin (belinda.martin@research.uwa.edu.au)

Abstract. Petroleum hydrocarbons (PHCs) are among the most prevalent sources of environmental contamination. It has been
15 hypothesized that plant root exudation of low molecular weight organic acid anions (carboxylates) may aid degradation of PHCs by stimulating heterotrophic microbial activity. We, therefore, applied two commonly-exuded carboxylates (citrate and malonate) to uncontaminated and diesel contaminated microcosms (10,000 mg kg⁻¹; aged 40 days) to determine their impact on the microbial community and PHC degradation. Every 48 hours for 18 days, soil received 5 μmol g⁻¹ of i) citrate, ii) malonate, iii) citrate + malonate or iv) water. Microbial activity was measured daily as the flux of CO₂. After 18 days, changes
20 in the microbial community were assessed by community level physiological profiles and 16S rRNA bacterial community profiles determined by denaturing gradient gel electrophoresis. Saturated PHCs remaining in the soil were assessed by GC-MS. Cumulative soil respiration increased four- to six-fold with the addition of carboxylates, while diesel contamination resulted in a small, but similar, increase across all carboxylate treatments. The addition of carboxylates resulted in distinct changes to the microbial community, but only a small decrease in the *n*-C17: pristane biomarker. We conclude that carboxylate
25 addition can increase microbial activity and modify the microbial community in both uncontaminated and diesel-contaminated soils. The impact of these changes on PHC biodegradation and rhizosphere processes, more generally, merits further research.

Key words: CLPP; carboxylates; organic acids; petroleum hydrocarbons; phytoremediation; rhizosphere

1. Introduction

30 Contamination of soils by petroleum hydrocarbons (PHCs) can impair soil function and pose serious risks to human and ecosystem health (Park and Park, 2011; Ramadass et al., 2015). A novel approach for remediating surface soils contaminated with PHCs is to grow plants in order to benefit from their stimulating effect on rhizosphere microorganisms (Ma et al., 2010).



Plant roots provide favorable conditions for rhizosphere microorganisms largely through the exudation of substrates that allow increased growth and activity (*e.g.* amino acids, carbohydrates and carboxylates). It has been speculated this increase in microbial growth and activity, that is driven by root exudates, will accelerate the rate of PHC biodegradation within the rhizosphere (Anderson et al., 1993; Nie et al., 2011; Phillips et al., 2012; Shahsavari et al., 2015). For instance, root exudates
5 have been linked to enhanced PHC degradation for plants grown *in situ* (Joner and Leyval, 2003; Gao et al., 2011) and in batch experiments in which exudates collected from plant roots were added to contaminated soils (Miya and Firestone, 2001; Yoshitomi and Shann, 2001; Xie et al., 2012).

Low molecular weight (LMW; molecular weight <500 MW) aliphatic organic acid anions (carboxylates) comprise a significant
10 proportion of root exudate profiles (Jones, 1998; Ryan et al., 2001). Carboxylates may act as biostimulating agents in the degradation of PHCs through the provision of a labile carbon source that supports increased microbial growth and activity. When added to soils, carboxylates are rapidly degraded by soil microorganisms, with up to 80% mineralized to CO₂ within 24 hours, depending upon the carboxylate added and the soil type (Evans, 1998; Ström et al., 2001; Hashimoto, 2007; Oburger et al., 2009; Fujii et al., 2010; Ryan et al., 2012). In addition, carboxylates have the capacity to enhance soil phosphorus supply
15 and, hence, microbial growth and activity through phosphate desorption either due to anion exchange or their ability to chelate to metal cations (*e.g.* Al³⁺, Fe³⁺ and Ca²⁺) (Jones and Darrah, 1994; Ryan et al., 2001; Shane and Lambers, 2005; Martin et al., 2014). Carboxylates may also function to increase the bioavailability of PHCs by promoting their desorption from the soil matrix (Ling et al., 2009; An et al., 2010, 2011; Gao et al., 2010; Keiluweit et al., 2015;), although this would depend on soil type and may require higher carboxylate concentrations than reportedly occur in soils (Ryan et al., 2001).

20 In addition to enhancing microbial growth and activity, root exudates such as carboxylates, may affect microbial community function by altering either gene expression, metabolic status and/or by selecting for the growth of specific microorganisms (Benizri et al., 2002; Baudoin et al., 2003; Butler et al., 2003; Hartmann et al., 2009; Louvel et al., 2011; Yergeau et al., 2014). For example, the structure of microbial communities associated with the cluster roots of white lupin (*Lupinus albus* L.) were
25 highly correlated to the level of carboxylate exudation (Marschner et al., 2002) and the addition of oxalate to an artificial rhizosphere environment caused more pronounced shifts in the microbial community than the addition of glucose (Keiluweit et al., 2015). The presence of PHCs is also likely to strongly influence the soil microbial community (Reddy et al., 2011; Hou et al., 2015). However, it is not known whether in a PHC-contaminated soil the addition of carboxylates will also alter microbial community composition.

30 The objective of this study was to determine the impact of two carboxylates commonly exuded by plant roots, citrate (a tri-carboxylate) and malonate (a di-carboxylate), on microbial activity and community structure in uncontaminated and diesel-contaminated soils. In the diesel-contaminated soil, the impact of carboxylate addition on the degradation of PHCs was also examined. We chose to use a microcosm approach. This enabled assessment of the impact the carboxylate addition in the



absence of confounding effects due to the presence of plant roots (*e.g.* PHC uptake/sorption, plant respiration, presence of other exudates). We hypothesized that; i) addition of carboxylates would enhance microbial activity (soil respiration); ii) addition of diesel and carboxylates would lead to shifts in the microbial community structure and function, and iii) addition of carboxylates would enhance degradation of PHCs.

5 2. Materials and methods

2.1 Soil characteristics

A loamy sand (~5% clay), previously uncontaminated by PHCs, was collected from the top mineral horizon (0–10 cm) of a pasture on a dairy farm 90 km south of Perth, Western Australia (32°45'31.16S, 115°49'33.88E). The soil was passed through a 2 mm mesh, stored in cloth bags and kept field moist at 4 °C until used. The soil contained 16 mg kg⁻¹ of NH₄-N and 121 mg kg⁻¹ of NO₃-N (Searle, 1984), 52 mg kg⁻¹ of bicarbonate-extractable P and 61 mg kg⁻¹ of bicarbonate-extractable K (Colwell, 1963), 88 mg kg⁻¹ of KCl-extractable S (Blair et al., 1991) and 3.3% organic C (Walkley and Black, 1934); pH_(CaCl2) was 4.6 and electrical conductivity was 34 mS m⁻¹. Soil water retention was determined after equilibrating saturated soil at a series of gas pressures (–0.1, –10, –33 and –100 kPa).

2.2 Experimental design

15 Previously uncontaminated soil was housed in gas-tight glass microcosms (385 mL). Soil in half the microcosms was spiked with 10,000 mg kg⁻¹ of diesel (obtained from a Caltex Inc. commercial bowser). Sterile deionized water was added to the microcosms to 40% of water holding capacity to negate moisture limitation (Tibbett et al., 2011). The soil was mixed thoroughly and incubated in the dark at 24 °C for 40 days. To prevent the build-up of volatiles and to allow gas exchange, microcosms were regularly aerated under a laminar flow and watered to weight to maintain the water holding capacity at 40%.

20

Stock solutions of citric and malonic acid solutions were prepared and the pH adjusted to 4.5 with KOH to ensure that the organic acids were predominantly in anionic form (Jones and Darrah, 1995). Every 48 hours, both diesel-contaminated and uncontaminated soils received either: i) citrate (5 μmol g⁻¹ soil), ii) malonate (5 μmol g⁻¹ soil), iii) citrate + malonate (2.5 μmol g⁻¹ soil per carboxylate), or iv) sterile deionized water (Table S1). Carboxylates were added for 18 days, equating to a total of 25 45 μmol g⁻¹ soil. There were four replicates in each treatment. Microcosms were incubated in the dark at 24 °C when not in use.

2.3 Soil respiration

Soil respiration was measured daily for 18 days on all microcosms using an infrared gas analyzer (Series 225 Gas Analyzer, Hoddesdon, UK) as previously described (Clegg et al., 1978). Measurement of CO₂ respiration is a common indicator for 30 measuring changes in microbial activity and has been shown to directly correlate with the extent of PHC degradation (Baptista



et al., 2005; Greenwood et al., 2009). Briefly, a sample of headspace gas (1 mL) was collected with a syringe from each microcosm and injected into the gas analyzer sample line. All samples were calibrated against a CO₂ standard (5.08% v/v). Following CO₂ analysis, the headspace of each microcosm was refreshed by airing with a fan for 20 seconds.

2.4.1 Post incubation measures

5 The experiment was halted after 18 days of carboxylate addition. Subsamples from three replicates of each treatment combination were removed for measurement of soil pH_(CaCl₂), as described previously, and determination of the community level physiological profile (commonly referred to as ‘CLPP’). Microbial community analysis using 16S rRNA bacterial community profiles determined by denaturing gradient gel electrophoresis (DGGE) was performed on two replicates of the diesel-contaminated treatments and the water control in the uncontaminated treatment. Saturated hydrocarbon analysis was
10 performed on three replicates of the diesel-contaminated treatment.

2.4.2 Community level physiological profile

CLPPs were determined using the MicroResp™ micro-plate system as described by Campbell et al. (2003). MicroResp is a rapid, culture-independent technique which allows assessment of active members of the microbial community based on their use of various carbon substrates (Campbell et al., 2003). Thirty-one known rhizosphere or root-exuded carbon substrates
15 (Campbell et al., 1997; Degens and Harris, 1997; Banning et al., 2012) were dissolved in water and added to deep-well plates to make a final concentration of 30 mg C g soil water⁻¹ (Table S2). The addition of each carbon substrate was replicated three times randomly throughout the plate. Soil (~0.23 g) was added to each well and the plates were then sealed and covered with a detection plate containing 150 µL Cresol Red agar (1% Noble Agar, 20 µg mL⁻¹ Cresol Red, 240 mM KCl and 4 mM NaHCO₃). The assembled system was secured with clamps and incubated in the dark for 4 hours at 24 °C. Carbon dioxide was
20 measured based upon the colorimetric reaction that occurs in the detection plate due to the change in pH as CO₂ reacts with bicarbonate. The absorbance for each detection plate was measured before (pre-assay) and after (post-assay) at 590 nm in a micro-plate reader (Multiskan, Thermo Scientific) using Skan-it software (version 2.2, Thermo Scientific). Post-assay absorbance values were normalized against pre-assay absorbance and converted to headspace CO₂ using a calibration curve obtained with detachable ‘combi strip’ dye wells (8 wells each) exposed to known volumes of standard CO₂ in sealed test tubes
25 (first flushed with N₂) for 4 hours at 24 °C.

2.4.3 Microbial community analysis

Soil DNA was extracted using a PowerSoil® DNA Isolation Kit (MoBio laboratories, Inc. USA) according to the manufacturer's guidelines. The soil bacterial community was assessed by PCR using universal primers 341F-GC and 917R on 16S rRNA genes. All PCR products were examined using 1.5% agarose gel electrophoresis prior to DGGE analysis (Simons
30 et al., 2012). DGGE was carried out using the Universal Mutation Detection System (BioRad) with a 6% urea-formamide



denaturant gradient polyacrylamide (40–60% denaturing gradient). The gel was run at 60 °C and 60 V for 18 hours, silver stained as described previously (Simons et al., 2012) and scanned using an Epson V700 scanner.

2.4.4 Saturated petroleum hydrocarbon analysis

Accelerated Solvent Extraction (ASE 200[®]) was used to extract remaining saturated PHCs from the soil. Soil (5 g) was mixed with diatomaceous earth (1:1), packed into a 33 mL extraction cell and extracted with 50% acetone and 50% hexane at 200 °C and 1500 psi, with a static time of 5 min, a flush volume of 60% and a purge time of 60 seconds. A soil sample freshly spiked with diesel fuel (10,000 mg kg⁻¹ of diesel) was also extracted at this time. The saturated PHCs were then separated using small column chromatography and eluted with *n*-pentane (Bastow et al., 2007). *N*-tetracosane was added as an internal standard. An aromatic fraction was subsequently eluted with *n*-pentane/dichloromethane (7:3 v/v), but for technical reasons could not be analyzed. The analysis of the saturated fraction was performed with an Agilent 7890A GC system using an HP-5 MS Agilent column (length 30 m, internal diameter 250 µm, film thickness 0.25 µm). Soil freshly spiked with diesel was also analyzed. The oven was programmed for 40 °C for 3 minutes then increased by 15 °C min⁻¹ to 320 °C with a total run time of 26 minutes and a helium carrier gas flow of 1.0 mL min⁻¹. MS conditions were ionization energy of 70 eV and source temperature of 230 °C. Product identifications were based on the retention profile and correlation to the internal standard. Peak areas were integrated using Agilent Enhanced ChemStation software (E.02.02.1431). Biodegradation was assessed by two commonly-used biomarker ratios, *n*-C17:pristane and *n*-C18:phytane, that were first normalized by the internal standard (Greenwood et al., 2008).

2.5 Statistical analysis

A two-way analysis of variance (ANOVA) was performed using Genstat version 14.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, Harpenden, UK) to determine the effect of carboxylate addition (water control, malonate, citrate + malonate, citrate) and diesel contamination (absent, present) upon cumulative soil respiration and soil pH. A one-way ANOVA was used to determine the effect of carboxylate addition on the biodegradation of saturated PHCs in the diesel-contaminated soil. The least significant difference at $P = 0.05$ is reported for significant main effects or interactions. Data were log transformed when required to satisfy the linear model assumptions.

CLPP data were first standardized by subtracting the control substrate (CO₂ response from cells containing only water) within each treatment and transformed to achieve normality using a fourth root transformation. A resemblance matrix (Table S3) was created using Euclidean distance and differences among *a priori* defined groups were examined by permutational multivariate analysis of variance (PERMANOVA) using Primer (version 6.1, PRIMER-E) and presented in an ordination using principal coordinates (PCO) (Anderson, 2001; Anderson and Willis, 2003). Pairwise comparisons were performed to determine whether CLPP data differed significantly among treatment combinations. Canonical analysis of principal coordinates (CAP) was also performed (Anderson and Willis, 2003).



In addition, CLPP data were divided into four main classes (amino acids [n = 6 substrates], aromatics [n = 6 substrates], carbohydrates [n = 3 substrates] and carboxylates [n = 12 substrates]: see Table S2.). Each class was then analyzed separately with a three-way ANOVA to determine the effect of carboxylate addition, diesel contamination and carbon substrate on CO₂ evolution during the four hours of the CLPP assay.

5

The DGGE gel was subject to analysis with Phoretix 1D software (Phoretix Ltd, UK). The similarities between the communities based on presence/absence of bands were expressed using the unweighted pair group method using arithmetic averages (UPGMA).

3. Results

10 3.1 Microbial activity

Cumulative CO₂ evolved over the 18-day incubation was increased by carboxylate addition ($P < 0.001$) and diesel contamination ($P < 0.001$), but there was no interaction between these two factors (Fig. 1). The addition of carboxylates increased cumulative CO₂ up to six-fold compared with the water control. Addition of malonate resulted in lower CO₂ evolution than addition of citrate + malonate or citrate (estimated means: malonate = 1.02, citrate + malonate = 1.33, citrate = 1.45, 15 LSD_{0.05} = 0.09). Diesel contamination resulted in a small increase in cumulative CO₂ evolved in all carboxylate treatments (estimated means: uncontaminated = 0.90, diesel contamination = 1.11, LSD_{0.05} = 0.06).

(Fig. 1)

20 While respiration was only measured every 48 hours in this experiment, it was recorded more frequently in a preliminary experiment (Fig. S1). In this preliminary experiment, by one hour after addition, the respiration rate had increased up to 16-fold for citrate and four-fold for malonate; it then halved by three hours after addition, remained steady until 24 hours after addition and then declined until reaching control levels by 74 hours.

3.2 Microbial community structure and function

25 3.2.1 Community level physiological profiles

Principal coordinates analysis (PCO) revealed a separation based on the microbial CLPPs among soils that received carboxylates and the water controls (Fig. 2; Table S3). The initial three PCO axes together accounted for 67.5% of the variation in the data (Fig. 2A). PERMANOVA of the CLPP data found an effect of carboxylate addition ($P_{\text{perm}} = 0.004$) with malonate ($P_{\text{perm}} = 0.002$) and citrate ($P_{\text{perm}} = 0.001$), but not citrate + malonate, as compared to the water control (Fig. 2A). There was 30 no effect of diesel contamination and no interaction between carboxylate addition and diesel contamination.



(Fig. 2)

Canonical analysis of the CLPP data, where the number of treatments and replicate structure is specified into *a priori* defined
5 groups, showed a clear difference among the three treatments when carboxylates were added and the water controls, again
irrespective of whether soils were diesel contaminated or not (Fig. 2B).

To further understand these utilization patterns, the 31 carbon substrates were divided into four main carbon groups (amino
acids, aromatics, carbohydrates, carboxylates; Table S2) and for each group the effect of carboxylate addition, diesel
10 contamination and individual substrate was examined using a three-way ANOVA (Table 1; Fig. 3). The carboxylate treatment
affected the utilization of all four classes of compounds. Utilization of amino acids, aromatics and carboxylates was greatly
enhanced in soil which had carboxylates applied previously, while for carbohydrates there was a complex three-way interaction
(results not shown). Diesel contamination affected the utilization of amino acids and carboxylates only. For amino acids, diesel
contamination reduced the utilization only in the citrate + malonate treatment. For the carboxylates, utilization was always
15 lower in the diesel-contaminated soil.

(Fig. 3)

(Table 1)

3.2.2 Microbial community analysis

20 DGGE of 16S rRNA gene bacterial community profiles showed clear differences among treatments (Fig. 4). Bacterial
communities receiving only water distinctly differed between uncontaminated and diesel-contaminated soils. However, a
greater differentiation occurred with the addition of carboxylates with the soils that received carboxylates (all diesel-
contaminated) strongly grouped compared with the water controls from the uncontaminated and diesel-contaminated soils.
Within the diesel-contaminated soil, the citrate + malonate treatment was more closely related to citrate than to malonate. The
25 water control in the diesel-contaminated soil showed two distinct bands that were much less distinct in the carboxylate
treatments and in the water control in the uncontaminated soil.

(Fig. 4)

3.3 Soil pH

30 For soil pH there was an interaction between the carboxylate treatment and the diesel-contamination treatment ($P = 0.001$)
(Table 2). This interaction was a result of the diesel contamination increasing pH only in the water control and malonate



treatment. The carboxylate treatment caused pH to increase in the order of citrate \geq citrate + malonate > malonate > water control.

(Table 2)

5 3.4 Degradation of saturated petroleum hydrocarbons

In the diesel-contaminated soil, the GC-MS chromatograms from the carboxylate addition treatments and the water control showed a reduction in saturated alkanes when compared to soil freshly spiked with diesel (Table 3). Total ion chromatograms of the carboxylate addition treatments and the water control showed a loss of small chain alkanes and an increasing prominence of unresolved complex mixture compared to the freshly spiked soil (data not shown). The biomarker *n*-C17:pristane was slightly, but significantly, reduced with the addition of carboxylates and the *n*-C18:phytane biomarker showed a similar trend, albeit not significant (Table 3). There was no significant difference in peak area of any of the individual saturated hydrocarbons with carboxylate addition (Table 3).

(Table 3)

15 4. Discussion

4.1 Carboxylates enhanced microbial activity in uncontaminated and diesel-contaminated soils

In the uncontaminated and diesel-contaminated soils, cumulative CO₂ evolution increased greatly in response to 18 days of repeated carboxylate addition compared to the water control. While some carboxylates may have been mineralized abiotically (such as may occur in the presence of Mn oxides), the relative contribution of this pathway is known to be minor (Jones et al., 1996). We, therefore, assume that in our study the increase in CO₂ evolution with addition of carboxylates was a result of carboxylates mineralization by the soil microbes. Thus, our first hypothesis that carboxylates enhance microbial activity was supported.

The rapid increase in soil respiration with the addition of carboxylates is in accordance with previous studies and is indicative of the ability of the soil microbial community to quickly mineralize carboxylates (Evans, 1998; van Hees et al., 2002; Jones et al., 1996). It implies a fast turnover rate of carboxylates in soils, which is consistent with van Hees et al. (2005b) who estimated a mean residence time for carboxylates of between 2 and 70 hours in the top mineral horizons, equating to a turnover of up to ten times a day (Van Hees et al., 2005b).

There was a clear difference in the ability of citrate and malonate to stimulate microbial activity; with citrate addition (either by itself or in combination with malonate) resulting in the highest cumulative CO₂ evolution in diesel-contaminated and



5 uncontaminated soils. Interestingly, malonate has been labeled a potential inhibitor of microbial respiration due to its inhibition of succinate dehydrogenase, a key enzyme of the citric acid cycle (Ikuma and Bonner, 1967; Li and Copeland, 2000; Phillips et al., 2012). However, our results show that malonate greatly increases soil respiration. This finding is consistent with that of Oburger et al. (2009) who found malonate did not inhibit microbial activity when added to a range of soil types in solutions also containing malate, citrate and oxalate.

4.2 Carboxylates impact soil microbial physiological profiles and community structure

The addition of carboxylates altered the microbial community in uncontaminated and diesel-contaminated soils, leading to the development of distinct communities as defined by the community level physiological profiles (CLPP) and the 16S rRNA bacterial community profiles (DGGE). The changes induced by the addition of carboxylates were complex and included, not surprisingly, an increased ability to use carboxylates, as well as amino acids and aromatics. Interestingly, the DGGE of the 16S rRNA bacterial community profiles showed two distinct bands in the diesel-contaminated water control which were less distinct when carboxylates had been added. Unfortunately, these bands were not sequenced so we do not know the identity of these microbes and whether they are known PHC degraders. However, the community shifts suggest that carboxylates have the potential to play a key role in shaping microbial community structure even in diesel-contaminated soils. Future studies incorporating 16S sequence analysis as well as functional gene analysis would help to uncover some of the key microbial players in these carboxylate-induced community shifts.

Soil pH also increased with carboxylate addition and it should be noted that pH can have a large influence on the composition of soil microbial communities (Fierer and Jackson, 2006). It is, therefore, possible that changes in soil pH are confounded with the effect of carboxylate addition on microbial community structure. Carboxylate addition has been shown to increase pH in the absence of plants and this has been attributed to microbial-mediated decarboxylation, as during decarboxylation a proton is consumed according to the following reaction: $R-CO-COO^- + H^+ \rightarrow R-CHO + CO_2$ (Yan et al., 1996; Rukshana et al., 2012; Keiluweit et al., 2015). The fact that soil pH was highest in the treatment with the greatest cumulative CO_2 evolution (citrate) is consistent with this scenario. However, the increase in soil pH may be due to the reaction of carboxylate ions with free H^+ in the soil solution, ammonification of organic nitrogen or NO_3^- uptake by microorganisms (Hinsinger et al., 2003).

4.3 Carboxylates did not significantly enhance the degradation of saturated PHCs over the course of 18 days

Loss of saturated hydrocarbons occurred in all treatments, including the water control, as shown by the reduction in saturated hydrocarbons compared to soil freshly spiked with diesel fuel. A large majority of these losses likely occurred before the addition of carboxylates due to initial volatilization of small chain hydrocarbons as the jars were vented. Carboxylate addition over 18 days did not have a major impact on the degradation of saturated hydrocarbons, as we only detected a slight change in one of the biodegradation biomarkers (*n*-C17:pristane) with the addition of carboxylates.



Carboxylates appear to have selected for microbes that are more stimulated by carboxylates than PHCs, leading to no major degradation of PHC's compared to the water control. Although no major significant differences were found, there was a trend of decreasing PHC abundance with addition of carboxylates over the time course of 18 days. Whether this trend would continue given a longer experimental time period is unknown. However, given phytoremediation trials often last longer than one year (Gurska et al., 2009; Vervaeke et al., 2003) the time frame of the current study may not have been long enough to detect significant declines.

Citrate, malate and oxalate have been shown to increase the desorption of phenanthrene and pyrene from various soils, however relatively high (up to 1000 mM *cf.* up to 600 μ M in rhizosphere) concentrations were used (Ling et al., 2009; An et al., 2010, 2011; Gao et al., 2010). The concentration of carboxylates applied in this study was chosen to be representative of the rhizosphere (e.g. 600 μ M) and may have been too low to have caused any significant desorption effects on the PHCs.

4.4 Limitations of this study and implications for bioremediation

The hypothesized links between enhanced PHC degradation in the rhizosphere and plant roots extend beyond the effects of a single carbon compound. Plant roots exude a wide array of compounds (*e.g.* amino acids, exo-enzymes, secondary metabolites) which, when released in localized areas, may act synergistically to both directly and/or indirectly enhance microbial PHC degradation (Kuiper et al., 2004; Martin et al., 2014). Therefore, the role of carboxylates in enhancing microbial PHC biodegradation may be greater in the presence of other root compounds (*e.g.* secondary metabolites) that enable a more diverse microbial community to be present. Additionally, in our study the experimental design and the techniques employed to reduce sample variability (*e.g.* root-free soil, constant rate of carboxylate supply, and sifting and mixing soil) are far from what would be experienced in a rhizosphere environment and so these results cannot be uncritically extrapolated to rhizoremediation studies performed *in situ*. Nonetheless, our findings provide noteworthy information pertaining to the potential effects of carboxylates on microbial community structure in soils in general and suggest that further investigation is warranted, particularly in regards to identifying particular microbial species or functional groups (PHC degraders and non-PHC degraders alike) that may be most influenced by carboxylate addition.

5. Conclusion

The results of this study suggest that carboxylates can stimulate microbial activity and alter microbial community structure in both uncontaminated and diesel-contaminated soils. However, we found no strong evidence to suggest that addition of carboxylates can enhance degradation of saturated hydrocarbons in the time frame of this study. However, our results suggest that carboxylates may have a role in determining microbial rhizosphere communities, even in PHC-contaminated soils.



6. Acknowledgements

This work was funded by an Australian Research Council linkage project (LP110201130) in partnership with Horizon Power, ChemCentre and Environmental Earth Sciences. CAP was supported by a Discovery Early Career Research Award from the Australian Research Council. Many thanks to Natalie Joyce, Daniel Ramsden, Daniel Kidd and Navjot Kaur for their technical
5 assistance.

References

- An, C., Huang, G., Yu, H., Wei, J., Chen, W. and Li, G.: Effect of short-chain organic acids and pH on the behaviors of pyrene in soil-water system, *Chemosphere*, 81, 1423–1429, doi:10.1016/j.chemosphere.2010.09.012, 2010.
- An, C., Huang, G., Wei, J. and Yu, H.: Effect of short-chain organic acids on the enhanced desorption of phenanthrene by
10 rhamnolipid biosurfactant in soil-water environment., *Water Res.*, 45, 5501–10, doi:10.1016/j.watres.2011.08.011, 2011.
- Anderson, M. J.: A new method for non-parametric multivariate analysis of variance, *Aust. Ecol.*, 26, 32–46, 2001.
- Anderson, M. J. and Willis, T. J.: Canonical analysis of principal coordinates : A useful method of constrained ordination for ecology, *Ecology*, 84, 511–525, 2003.
- Anderson, T. A., Guthrie, E. A. and Walton, B. T.: Bioremediation, *Environ. Sci. Technol.*, 27, 2630–2636, 1993.
- 15 Banning, N. C., Lalor, B. M., Cookson, W. R., Grigg, A. H. and Murphy, D. V.: Analysis of soil microbial community level physiological profiles in native and post-mining rehabilitation forest: Which substrates discriminate?, *Appl. Soil Ecol.*, 56, 27–34, doi:10.1016/j.apsoil.2012.01.009, 2012.
- Baptista, S. J., Cammarota, M. C., Dias, D. and Freire, D. C.: Production of CO₂ in crude oil bioremediation in clay soil, *Brazilian Arch. Biol. Technol.*, 48, 249–255, 2005.
- 20 Bastow, T. P., van Aarssen, B. G. K. and Lang, D.: Rapid small-scale separation of saturate, aromatic and polar components in petroleum, *Org. Geochem.*, 38, 1235–1250, doi:10.1016/j.orggeochem.2007.03.004, 2007.
- Baudoin, E., Benizri, E. and Guckert, A.: Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere, *Soil Biol. Biochem.*, 35, 1183–1192, doi:10.1016/S0038-0717(03)00179-2, 2003.
- Benizri, E., Dedourge, O., Dibattista-Leboeuf, C., Piutti, S., Nguyen, C. and Guckert, A.: Effect of maize rhizodeposits on
25 soil microbial community structure, *Appl. Soil Ecol.*, 21, 261–265, doi:10.1016/S0929-1393(02)00094-X, 2002.
- Blair, G. J., Chinoim, N., Lefroy, R. D. B., Anderson, G. C. and Crocker, G. J.: A soil sulfur test for pastures and crops, *Soil Res.*, 29, 619–626, 1991.
- Butler, J. L., Williams, M. A., Bottomley, P. J. and Myrold, D. D.: Microbial community dynamics associated with rhizosphere carbon flow, *Appl. Environ. Microbiol.*, 69, 6793–6800, doi:10.1128/AEM.69.11.6793, 2003.
- 30 Campbell, C. D., Grayston, S. J. and Hirst, D. J.: Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities., *J. Microbiol. Methods*, 30, 33–41, 1997.
- Campbell, C. D., Chapman, S. J., Cameron, C. M., Davidson, M. S. and Potts, J. M.: A rapid microtiter plate method to measure



- carbon dioxide evolved from carbon substrate amendments so as to determine the physiological profiles of soil microbial communities by using whole soil, *Appl. Environ. Microbiol.*, 69, 3593–3599, doi:10.1128/AEM.69.6.3593, 2003.
- Clegg, M. D., Sullivan, C. Y. and Eastin, J. D.: A sensitive technique for the rapid measurement of carbon dioxide concentrations, *Plant Physiol.*, 62, 924–926, 1978.
- 5 Colwell, J. D.: The estimation of the phosphorus fertilizer requirements of wheat in southern New South Wales by soil analysis, *Aust. J. Exp. Agric. Anim. Husb.*, 3, 190–197, 1963.
- Degens, B. P. and Harris, J. A.: Development of a physiological approach to measuring the catabolic diversity of soil microbial communities, *Soil Biol. Biochem.*, 29, 1309–1320, doi:10.1016/S0038-0717(97)00076-X, 1997.
- Evans, A.: Biodegradation of ¹⁴C-labeled low molecular organic acids using three biometer methods, *J. Geochemical Explor.*,
10 65, 17–25, doi:10.1016/S0375-6742(98)00058-2, 1998.
- Fierer, N. and Jackson, R. B.: The diversity and biogeography of soil bacterial communities., *Proc. Natl. Acad. Sci. U. S. A.*, 103, 626–631, doi:10.1073/pnas.0507535103, 2006.
- Fujii, K., Hayakawa, C., van Hees, P. A. W., Funakawa, S. and Kosaki, T.: Biodegradation of low molecular weight organic compounds and their contribution to heterotrophic soil respiration in three Japanese forest soils, *Plant Soil*, 334, 475–489,
15 doi:10.1007/s11104-010-0398-y, 2010.
- Gao, Y., Ren, L., Ling, W., Kang, F., Zhu, X. and Sun, B.: Effects of low-molecular-weight organic acids on sorption–desorption of phenanthrene in soils, *Soil Sci. Soc. Am. J.*, 74, 51, doi:10.2136/sssaj2009.0105, 2010.
- Gao, Y., Yang, Y., Ling, W., Kong, H. and Zhu, X.: Gradient distribution of root exudates and polycyclic aromatic hydrocarbons in rhizosphere soil, *Soil Sci. Soc. Am. J.*, 75, 1694, doi:10.2136/sssaj2010.0244, 2011.
- 20 Greenwood, P. F., Wibrow, S., George, S. J. and Tibbett, M.: Sequential hydrocarbon biodegradation in a soil from arid coastal Australia, treated with oil under laboratory controlled conditions, *Org. Geochem.*, 39, 1336–1346, doi:10.1016/j.orggeochem.2008.05.005, 2008.
- Greenwood, P. F., Wibrow, S., George, S. J. and Tibbett, M.: Hydrocarbon biodegradation and soil microbial community response to repeated oil exposure, *Org. Geochem.*, 40, 293–300, doi:10.1016/j.orggeochem.2008.12.009, 2009.
- 25 Gurska, K., Wang, W., Gerhardt, K., Khalid, A., Isherwood, D., Huang, X., Glick, B. and Greenberg, B.: Three year field test of a plant growth promoting rhizobacteria enhanced phytoremediation system at a land farm for treatment of hydrocarbon waste, *Environ. Sci. Technol.*, 43, 4472–4479, 2009.
- Hartmann, A., Schmid, M., van Tuinen, D. and Berg, G.: Plant-driven selection of microbes, *Plant Soil*, 321, 235–257, doi:10.1007/s11104-008-9814-y, 2009.
- 30 Hashimoto, Y.: Citrate sorption and biodegradation in acid soils with implications for aluminum rhizotoxicity, *Appl. Geochemistry*, 22, 2861–2871, doi:10.1016/j.apgeochem.2007.07.006, 2007.
- van Hees, P. A. W., Jones, D. L. and Godbold, D. L.: Biodegradation of low molecular weight organic acids in coniferous forest podzolic soils, *Soil Biol. Biochem.*, 34, 1261–1272, doi:10.1016/S0038-0717(02)00068-8, 2002.
- van Hees, P. A. W., Jones, D. L., Nyberg, L., Holmstrom, S. J. M., Godbold, D. L. and Lundstrom, U. S.: Modelling low



- molecular weight organic acid dynamics in forest soils., *Soil Biol. Biochem.*, 37, 517–531, doi:10.1016/j.soilbio.2004.08.014, 2005a.
- van Hees, P. A. W., Jones, D. L., Finlay, R., Godbold, D. L. and Lundstrom, U. S.: The carbon we do not see - the impact of low molecular weight compounds on carbon dynamics and respiration in forest soils: a review., *Soil Biol. Biochem.*, 37, 1–13, doi:10.1016/j.soilbio.2004.06.010, 2005b.
- Hinsinger, P., Plassard, C., Tang, C. and Jaillard, B.: Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review, *Plant Soil*, 248, 43–59, doi:10.1023/A:1022371130939, 2003.
- Hou, J., Liu, W., Wang, B., Wang, Q., Luo, Y. and Franks, A. E.: PGPR enhanced phytoremediation of petroleum contaminated soil and rhizosphere microbial community response, *Chemosphere*, 138, 592–598, doi:10.1016/j.chemosphere.2015.07.025, 2015.
- Ikuma, H. and Bonner, W. D.: Properties of higher plant mitochondria. III. Effects of respiratory inhibitors., *Plant Physiol.*, 42, 1535–1544, doi:10.1104/pp.42.11.1535, 1967.
- Joner, E. J. and Leyval, C.: Rhizosphere gradients of polycyclic aromatic hydrocarbon (PAH) dissipation in two industrial soils and the impact of arbuscular mycorrhiza, *Environ. Sci. Technol.*, 37, 2371–2375, doi:10.1021/es020196y, 2003.
- Jones, D.: Organic acids in the rhizosphere - a critical review, *Plant Soil*, 205, 25–44, 1998.
- Jones, D. L. and Darrah, P. R.: Role of root derived organic acids in the mobilization of nutrients from the rhizosphere, *Plant Soil*, 166, 247–257, doi:10.1007/BF00008338, 1994.
- Jones, D. L. and Darrah, P. R.: Influx and efflux of organic acids across the soil-root interface of *Zea mays* L. and its implications in rhizosphere C flow, *Plant Soil*, 173, 103–109, doi:10.1007/BF00155523, 1995.
- Jones, D. L., Prabowo, A. M. and Kochian, L. V.: Kinetics of malate transport and decomposition in acid soils and isolated bacterial populations: The effect of microorganisms on root exudation of malate under Al stress, *Plant Soil*, 182, 239–247, 1996
- Keiluweit, M., Bougoure, J. J., Nico, P. S., Pett-Ridge, J., Weber, P. K. and Kleber, M.: Mineral protection of soil carbon counteracted by root exudates, *Nat. Clim. Chang.*, 1–28, doi:10.1038/nclimate2580, 2015.
- Kuiper, I., Lagendijk, E. L., Bloembergen, G. V and Lugtenberg, B. J. J.: Rhizoremediation: A beneficial plant-microbe interaction bioremediation: A natural method, *Mol. Plant. Microbe. Interact.*, 17, 6–15, 2004.
- Li, J. and Copeland, L.: Role of malonate in chickpeas, *Phytochemistry*, 54, 585–589, doi:10.1016/S0031-9422(00)00162-X, 2000.
- Ling, W., Ren, L., Gao, Y., Zhu, X. and Sun, B.: Impact of low-molecular-weight organic acids on the availability of phenanthrene and pyrene in soil, *Soil Biol. Biochem.*, 41, 2187–2195, doi:10.1016/j.soilbio.2009.08.003, 2009.
- Louvel, B., Cébron, A. and Leyval, C.: Root exudates affect phenanthrene biodegradation, bacterial community and functional gene expression in sand microcosms, *Int. Biodeterior. Biodegrad.*, 65, 947–953, doi:10.1016/j.ibiod.2011.07.003, 2011.
- Ma, B., He, Y., Chen, H. H., Xu, J. M. and Rengel, Z.: Dissipation of polycyclic aromatic hydrocarbons (PAHs) in the rhizosphere: Synthesis through meta-analysis, *Environ. Pollut.*, 158, 855–861, doi:10.1016/j.envpol.2009.09.024, 2010.



- Marschner, P., Neumann, G., Kania, A., Weiskopf, L. and Lieberei, R.: Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.), *Plant Soil*, 246, 167–174, 2002.
- Martin, B. C., George, S. J., Price, C. A., Ryan, M. H. and Tibbett, M.: The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: Current knowledge and future directions, *Sci. Total Environ.*, 472, 642–653, doi:10.1016/j.scitotenv.2013.11.050, 2014.
- 5 Miya, R. K. and Firestone, M. K.: Enhanced phenanthrene biodegradation in soil by slender oat root exudates and root debris., *J. Environ. Qual.*, 30, 1911–1918, doi:10.2134/jeq2001.1911, 2001.
- Nie, M., Wang, Y., Yu, J., Xiao, M., Jiang, L., Yang, J., Fang, C., Chen, J. and Li, B.: Understanding plant-microbe interactions for phytoremediation of petroleum-polluted soil, *PLoS One*, 6, 1–8, doi:10.1371/journal.pone.0017961, 2011.
- 10 Oburger, E., Kirk, G. J. D., Wenzel, W. W., Puschenreiter, M. and Jones, D. L.: Interactive effects of organic acids in the rhizosphere, *Soil Biol. Biochem.*, 41, 449–457, doi:10.1016/j.soilbio.2008.10.034, 2009.
- Park, I. S. and Park, J. W.: Determination of a risk management primer at petroleum-contaminated sites: Developing new human health risk assessment strategy, *J. Hazard. Mater.*, 185, 1374–1380, doi:10.1016/j.jhazmat.2010.10.058, 2011.
- Phillips, L. A., Greer, C. W., Farrell, R. E. and Germida, J. J.: Plant root exudates impact the hydrocarbon degradation potential of a weathered-hydrocarbon contaminated soil, *Appl. Soil Ecol.*, 52, 56–64, doi:10.1016/j.apsoil.2011.10.009, 2012.
- 15 Ramadass, K., Megharaj, M. and Venkateswarlu, K.: Ecological implications of motor oil pollution : Earthworm survival and soil health, *Soil Biol. Biochem.*, 85, 72–81, doi:10.1016/j.soilbio.2015.02.026, 2015.
- Reddy, M. V., Devi, M. P., Chandrasekhar, K., Goud, R. K. and Mohan, S. V.: Aerobic remediation of petroleum sludge through soil supplementation: Microbial community analysis, *J. Hazard. Mater.*, 197, 80–87, doi:10.1016/j.jhazmat.2011.09.061, 2011.
- 20 Rukshana, F., Butterly, C. R., Baldock, J. a., Xu, J. M. and Tang, C.: Model organic compounds differ in priming effects on alkalinity release in soils through carbon and nitrogen mineralisation, *Soil Biol. Biochem.*, 51, 35–43, doi:10.1016/j.soilbio.2012.03.022, 2012.
- Ryan, M. H., Tibbett, M., Edmonds-Tibbett, T., Suriyagoda, L. D. B., Lambers, H., Cawthray, G. R. and Pang, J.: Carbon trading for phosphorus gain: The balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition, *Plant, Cell Environ.*, 35, 2170–2180, doi:10.1111/j.1365-3040.2012.02547.x, 2012.
- 25 Ryan, P. R., Delhaize, E. and Jones, D. L.: Function and mechanism of organic anion exudation from plant roots, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 52, 527–560, 2001.
- Searle, P. L.: The berthelot or indophenol reaction and its use in the analytical chemistry of nitrogen. A review., *Analyst*, 109, 549–568, 1984.
- 30 Shahsavari, E., Adetutu, E. M., Taha, M. and Ball, A. S.: Rhizoremediation of phenanthrene and pyrene contaminated soil using wheat, *J. Environ. Manage.*, 155, 171–176, doi:10.1016/j.jenvman.2015.03.027, 2015.
- Shane, M. W. and Lambers, H.: Cluster roots: A curiosity in context, *Plant Soil*, 274, 101–125, doi:10.1007/s11104-004-2725-7, 2005.



- Simons, K. L., Ansar, A., Kadali, K., Bueti, A., Adetutu, E. M. and Ball, A. S.: Investigating the effectiveness of economically sustainable carrier material complexes for marine oil remediation, *Bioresour. Technol.*, 126, 202–207, doi:10.1016/j.biortech.2012.09.053, 2012.
- Ström, L., Owen, A. G., Godbold, D. L. and Jones, D. L.: Organic acid behaviour in a calcareous soil: Sorption reactions and biodegradation rates, *Soil Biol. Biochem.*, 33, 2125–2133, doi:10.1016/S0038-0717(01)00146-8, 2001.
- 5 Tibbett, M., George, S. J., Davie, A., Barron, A., Milton, N. and Greenwood, P. F.: Just add water and salt: The optimisation of petrogenic hydrocarbon biodegradation in soils from semi-arid Barrow Island, Western Australia, *Water. Air. Soil Pollut.*, 216, 513–525, doi:10.1007/s11270-010-0549-z, 2011.
- Vervaeke, P., Luysaert, S., Mertens, J., Meers, E., Tack, F. M. G. and Lust, N.: Phytoremediation prospects of willow stands on contaminated sediment: A field trial, *Environ. Pollut.*, 126, 275–282, doi:10.1016/S0269-7491(03)00189-1, 2003.
- 10 Walkley, A. and Black, I. A.: An examination of the degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method., *Soil Sci.*, 37, 29–38, 1934.
- Xie, X. M., Liao, M., Yang, J., Chai, J. J., Fang, S. and Wang, R. H.: Influence of root-exudates concentration on pyrene degradation and soil microbial characteristics in pyrene contaminated soil, *Chemosphere*, 88, 1190–1195, doi:10.1016/j.chemosphere.2012.03.068, 2012.
- 15 Yan, F., Schubert, S. and Mengel, K.: Soil pH increase due to biological decarboxylation of organic anions, *Soil Biol. Biochem.*, 28, 617–624, doi:10.1016/0038-0717(95)00180-8, 1996.
- Yergeau, E., Sanschagrin, S., Maynard, C., St-Arnaud, M. and Greer, C. W.: Microbial expression profiles in the rhizosphere of willows depend on soil contamination, *ISME J.*, 8, 344–358, doi:10.1038/ismej.2013.163, 2014.
- 20 Yoshitomi, K. and Shann, J. : Corn (*Zea mays* L.) root exudates and their impact on ¹⁴C-pyrene mineralization, *Soil Biol. Biochem.*, 33, 1769–1776, doi:10.1016/S0038-0717(01)00102-X, 2001.

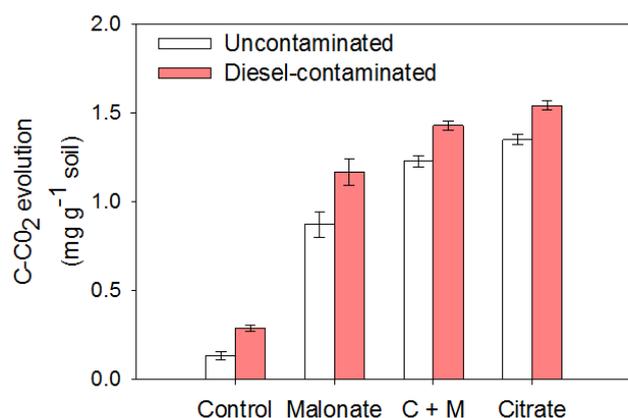


Figure 1: Cumulative CO₂ evolved over the 18-day incubation in diesel-contaminated and uncontaminated soils which received water (control) or 5 μmol g⁻¹ soil of citrate, malonate, or citrate + malonate every 48 hours. Values are means ± SE (n=4). There was an effect of carboxylate addition ($P < 0.001$) and diesel-contamination ($P < 0.001$), but no significant interaction.

5

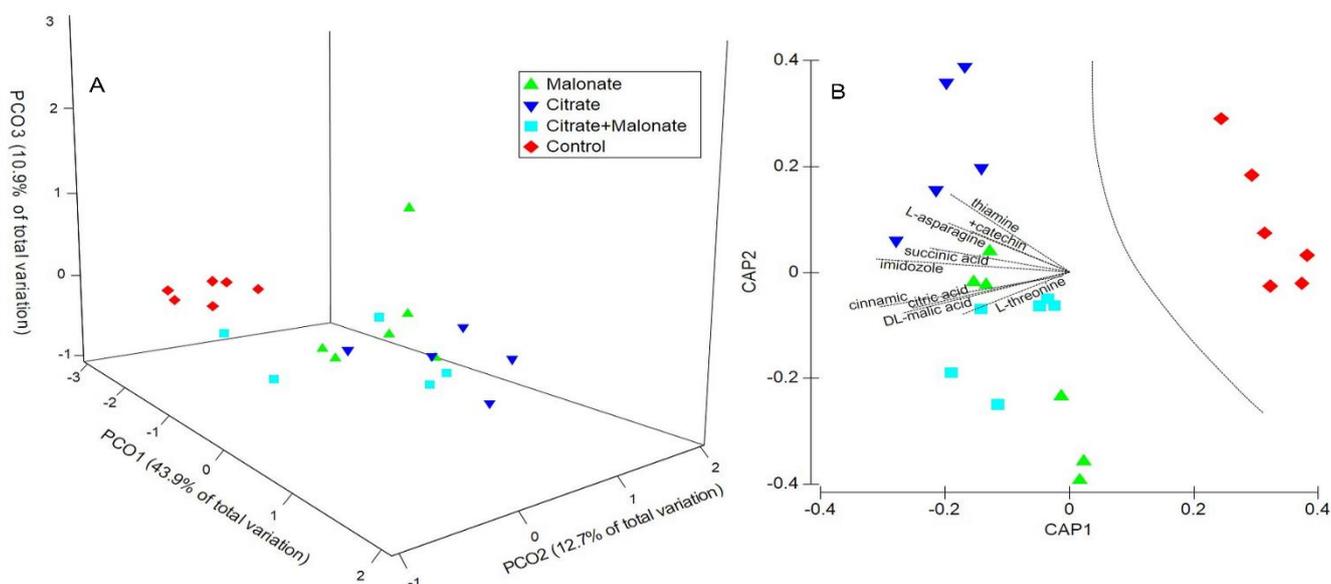
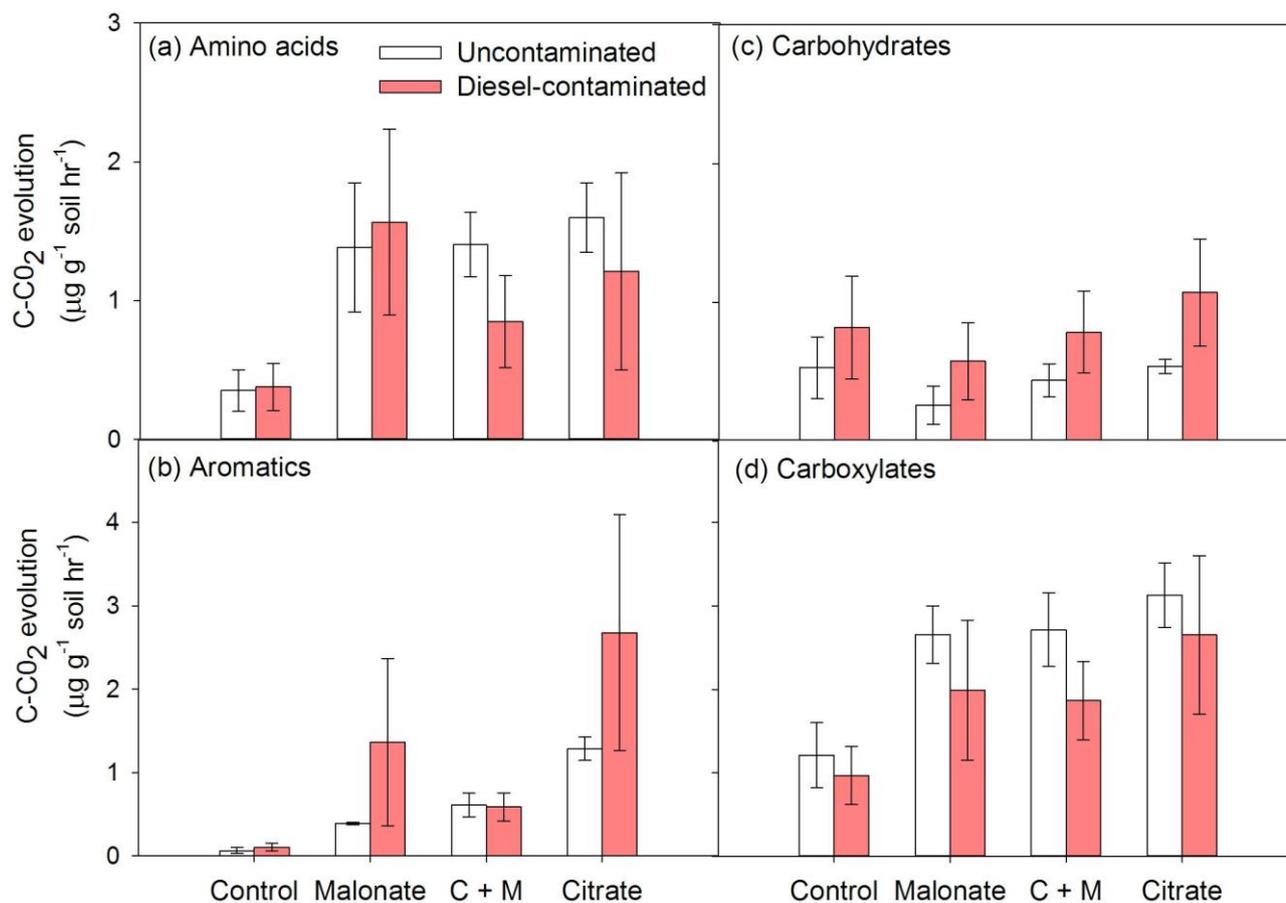
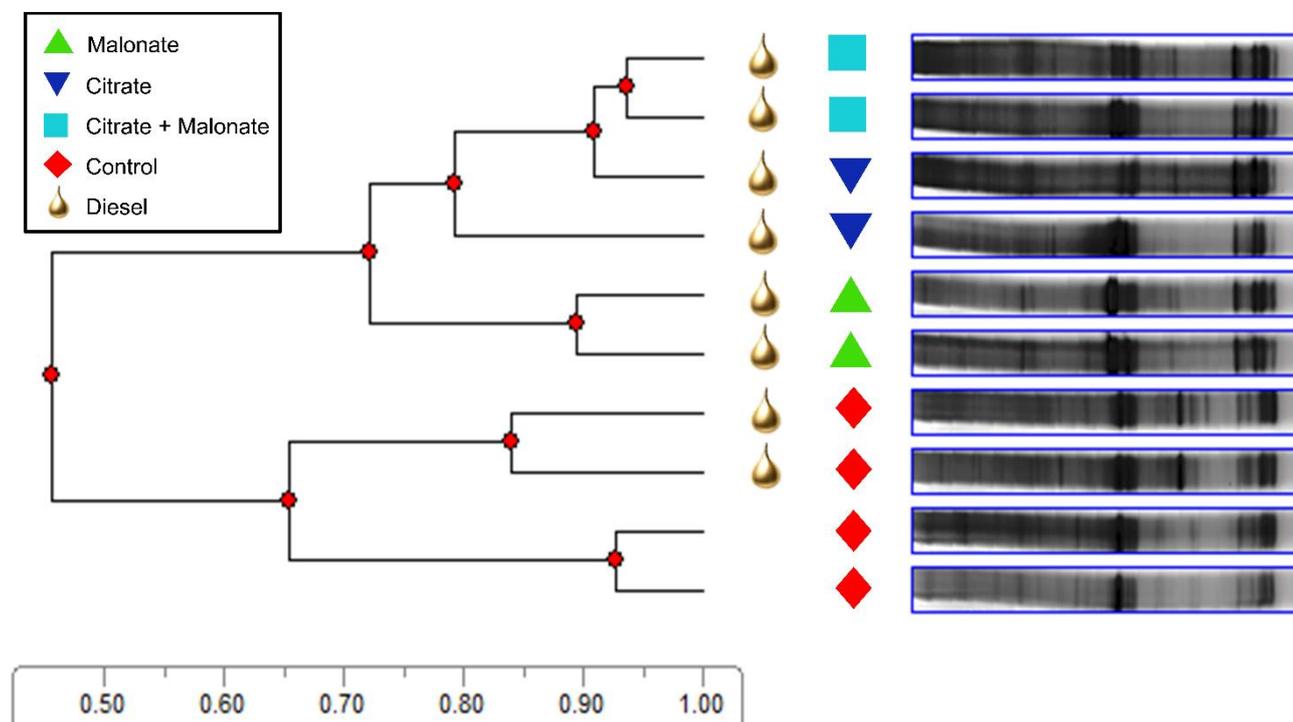


Figure 2: (a) Principal co-ordinate analysis ordination (PCO) and (b) canonical analysis of principal co-ordinates (CAP) of CLPP on soil after 18 days of incubation for diesel-contaminated and uncontaminated soils which received water (control) or 5 μmol g⁻¹ soil of citrate, malonate, or citrate + malonate every 48 hours. As there was no effect of diesel-contamination there is no distinction made between the three replicates of each carboxylate treatment from diesel-contaminated soil and the three replicates from uncontaminated soil. The curve in Fig. 2 (b) represents a significant difference ($P_{\text{Perm}} = 0.004$ based on PERMANOVA) between treatments receiving carboxylates and the water control.

10



5 **Figure 3: Estimated means for the interaction of carboxylate treatment and diesel contamination for CLPP on soil after 18 days of incubation for diesel-contaminated and uncontaminated soils which received water or 5 μmol g⁻¹ soil of malonate, citrate + malonate or citrate every 48 hours and was mixed with 27 substrates from four main classes (amino acids [n=6 substrates], aromatics [n=6 substrates], carboxylates [n=12 substrates], carbohydrates [n=3 substrates]). Values are means ± SEM (n=3). Statistical outcomes are presented in Table 3.**



5 **Figure 4:** UPGMA dendrogram of 16s rRNA DGGE bacterial community profiles after 18 days of incubation in diesel-contaminated and uncontaminated soil which received water (control) and diesel-contaminated soil that received 5 $\mu\text{mol g}^{-1}$ soil of malonate, citrate + malonate or citrate every 48 hours. The scale represents the similarity coefficient.

10

15



Table 1. Outcomes of a three-way analysis of variance on the community level physiological profiles (CLPPs) after an 18-day incubation of diesel-contaminated and uncontaminated soils which received water or 5 $\mu\text{mol g}^{-1}$ soil of malonate, citrate + malonate or citrate every 48 hours.

	Carboxylate addition (C)	Diesel contamination (D)	Substrate (S)	C \times D	C \times S	D \times S	C \times D \times S
Substrate class							
Amino acids (n = 6)	<0.001	0.02	0.046	0.013	n.s.*	n.s.	n.s.
Aromatics (n = 6)	<0.001	n.s.	<0.001	n.s.	n.s.	n.s.	n.s.
Carbohydrates (n = 3)	n.s.	n.s.	<0.001	n.s.	n.s.	n.s.	0.041
Carboxylates (n = 12)	<0.001	0.001	<0.001	n.s.	n.s.	n.s.	n.s.

* n.s. = not significant

5

10

15

20



5

Table 2. Soil $\text{pH}_{(\text{CaCl}_2)}$ after an 18-day incubation of diesel-contaminated and uncontaminated soils which received water or $5 \mu\text{mol g}^{-1}$ soil of malonate, citrate + malonate or citrate every 48 hours. Values are means \pm SE ($n = 3$). There was an interaction between carboxylate addition and diesel contamination ($P = 0.001$, $\text{LSD}_{0.05} = 0.055$). Initial soil pH was 4.6 and carboxylate solution pH was 4.5.

	Uncontaminated soil	Diesel-contaminated soil
Control	4.47 ± 0.01	4.67 ± 0.02
Malonate	6.64 ± 0.01	6.78 ± 0.01
Citrate + malonate	7.18 ± 0.01	7.23 ± 0.02
Citrate	7.29 ± 0.01	7.31 ± 0.04

10

15

20

25



Table 3. Saturated hydrocarbons in a soil freshly spiked with diesel and after an 18-days incubation of diesel-contaminated soils which received water or 5 $\mu\text{mol g}^{-1}$ soil of malonate, citrate + malonate, or citrate every 48 hours. Values are standardized mean peak area \pm SE (n = 3).

	Hexadecan e (n-C16)	Heptadeca ne (n-C17)	Octadecane (n-C18)	Norpristan	Pristane	Phytane	n-C17:Pr	n-C18:Ph	Pr:Ph
Freshly spiked soil	8.521	8.526	7.590	2.828	4.363	2.767	1.954	2.744	1.577
Treatment									
Control	0.274 \pm 0.408	\pm 0.276	\pm 0.763	\pm 1.823	\pm 1.376	\pm 0.225	\pm 0.201	\pm 1.322	\pm 0.038
Malonate	0.021	0.047	0.035	0.098	0.267	0.149	0.006	0.010	0.038
	0.190 \pm 0.345	\pm 0.231	\pm 0.739	\pm 1.882	\pm 1.365	\pm 0.182	\pm 0.168	\pm 1.378	\pm 0.010
	0.096	0.081	0.058	0.093	0.139	0.102	0.019	0.018	0.010
Citrate malonate	0.227 \pm 0.376	\pm 0.241	\pm 0.726	\pm 1.951	\pm 1.421	\pm 0.191	\pm 0.169	\pm 1.377	\pm 0.038
	0.076	0.081	0.068	0.140	0.269	0.224	0.009	0.019	0.038
Citrate	0.228 \pm 0.362	\pm 0.239	\pm 0.657	\pm 1.951	\pm 1.437	\pm 0.184	\pm 0.163	\pm 1.358	\pm 0.004
	0.105	0.097	0.112	0.159	0.242	0.175	0.016	0.033	0.004
ANOVA outcomes									
Effect treatment	n.s.**	n.s.	n.s.	n.s.	n.s.	n.s.	P = 0.029	n.s.	n.s.
LSD _{0.05}							0.03		

* Pr = pristane and Ph = phytane. **n.s. = not significant